Published in final edited form as:

Pain. 2017 September ; 158(9): 1647–1655. doi:10.1097/j.pain.0000000000000953.

Early life vincristine exposure evokes mechanical pain hypersensitivity in the developing rat

Katie A. Schappacher^{a,b}, Lauren Styczynski^a, and Mark L. Baccei^a

^aPain Research Center, Department of Anesthesiology, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati OH 45267, USA

^bDepartments of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati OH 45267, USA

Abstract

Vincristine (VNC) is commonly used to treat pediatric cancers, including the most prevalent childhood malignancy, Acute Lymphoblastic Leukemia. While clinical evidence suggests that VNC causes peripheral neuropathy in children, the degree to which pediatric chemotherapeutic regimens influence pain sensitivity throughout life remains unclear, in part due to the lack of an established animal model of chemotherapy-induced neuropathic pain during early life. Therefore, the present study investigated the effects of VNC exposure between postnatal days (P) 11 and 21 on mechanical and thermal pain sensitivity in the developing rat. Low doses of VNC (15 or 30 µg/kg) failed to alter nociceptive withdrawal reflexes at any age examined compared to vehicle-injected littermate controls. Meanwhile, high dose VNC (60 µg/kg) evoked mechanical hypersensitivity in both sexes beginning at P26 that persisted until adulthood, and included both static and dynamic mechanical allodynia. Hindpaw withdrawal latencies to noxious heat and cold were unaffected by high doses of VNC, suggesting a selective effect of neonatal VNC on mechanical pain sensitivity. Gross and fine motor function appeared normal after VNC treatment, although a small decrease in weight gain was observed. The VNC regimen also produced a significant decrease in intraepidermal nerve fiber (IENF) density in the hindpaw skin by P33. Overall, the present results demonstrate that high dose administration of VNC during the early postnatal period selectively evokes a mechanical hypersensitivity that is slow to emerge during adolescence, providing further evidence that aberrant sensory input during early life can have prolonged consequences for pain processing.

1. Introduction

Advances in the clinical treatment of pediatric cancers have produced a dramatic increase in survival rates [49]. For example, the five-year survival rate for Acute Lymphoblastic Leukemia (ALL), the most common childhood malignancy with a peak onset between 2–3 years of age, has increased from 60% in 1975 to ~90% today [24,51]. Current chemotherapy regimens for ALL commonly include the vinca alkaloid derivative vincristine (VNC)

Corresponding Author: Dr. Mark L. Baccei, Pain Research Center, Dept. of Anesthesiology, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati OH 45267, mark.baccei@uc.edu.

The authors declare that they have no conflicts of interest with any of the work presented in this manuscript.

[38,56,60]. Both adult and pediatric patients receiving tubulin-binding drugs can experience symptoms of sensory and motor peripheral neuropathy including pain and paresthesia in the hands and/or feet, as well as a loss of deep tendon reflexes [28,37,73], which often leads to dose reduction and negatively impacts the patients' quality of life.

Preclinical studies have clearly demonstrated that pain pathways are organized in a fundamentally different manner during the early postnatal period [19,20,35], which influences their response to tissue or nerve injury. For example, neonatal tissue damage can evoke unique changes in nociceptive processing within both the peripheral [6,18,32] and central nervous systems [39–41,67]. In addition, the effects of peripheral nerve injury in the rodent are highly age-dependent [21,29,45], with the onset of neuropathic pain delayed in the neonate due to a significant developmental shift in spinal neuroimmune signaling towards a more pro-inflammatory phenotype during adolescence [4,43].

While clinical evidence supports a link between early life VNC exposure and pain hypersensitivity, the degree to which the severity of pain resulting from chemotherapy-induced peripheral neuropathy (CIPN) changes with age remains unclear. Retrospective studies include a wide range of ages at diagnosis as well as variations in the chosen chemotherapy agents and cumulative dosage, making it difficult to rigorously assess the effects of vincristine on nociceptive processing at specific ages. Meanwhile, to our knowledge, long-term prospective studies using quantitative sensory testing (QST) approaches [78] to longitudinally measure nociceptive sensitivity in pediatric cancer survivors during adolescence or adulthood have yet to be undertaken. An animal model could yield valuable insight into the consequences of pediatric chemotherapeutic regimens for pain sensitivity throughout life. Although CIPN models have been well-established in adult rodents [1,3,46,72], the known developmental changes in pain circuits make it difficult to extrapolate the prior findings to the pediatric population.

Therefore, the present study investigated the effects of early life exposure to vincristine on mechanical and thermal pain sensitivity in the developing rat, with the goal of establishing a preclinical model of pediatric CIPN. Our results demonstrate that VNC administration during the early postnatal period evokes a selective increase in mechanical pain sensitivity that persists into adulthood. This work lays a foundation for the future exploration of the neurobiological mechanisms underlying CIPN in children and adolescents.

2. Methods

2.1 Subjects

Male and female Sprague Dawley rats (n = 108; Harlan, Indianapolis, IN) were used in this study. Animals were randomly assigned to one of two groups: vehicle (n = 54) or vincristine (n = 54). During treatment, animals were weighed prior to each injection then once per week after treatment ended. Behavioral assays were performed on separate days to avoid stress induced by multiple tests, with a minimum of 24 hours between assays in a given animal. All rats underwent a combination of tests for mechanical and thermal sensitivity as well as motor function, although an individual animal was not subjected to all seven behavioral assays (von Frey hairs, paintbrush, pinprick, Hargreaves test; cold plantar assay, rotarod and

foot fault test; see below) to again minimize the level of stress induced over the course of postnatal development. All experiments adhered to animal welfare guidelines established by the University of Cincinnati Institutional Animal Care and Use Committee.

2.2 Drug administration

Preliminary dose response studies consisted of 9 total i.p. injections of vincristine (15, 30, 45, or 60 µg/kg dissolved in sterile saline; Tocris) with a four-day on, two-day off, five-day on schedule starting on postnatal day (P)11. Since daily administration of 45 or 60 µg/kg vincristine (VNC) led to death after the third consecutive dose, we modified the dosing schedule to consist of 5 total injections of 60 µg/kg VNC on P11, 13, 17, 19 and 21 [3,72]. While drawing exact parallels between the age of rats and humans is inherently difficult, prior work has estimated the neurodevelopmental stage of a P7 rat as roughly equivalent to that of a human born at full-term [13]. P11 was chosen as a starting point in the present study in order to: (1) better reflect the clinical scenario in which vincristine is most commonly administered to children between the ages of 2 and 3 rather than to newborns [51]; and (2) facilitate the comparison of our findings with those of prior studies of infant neuropathic pain which employed mechanical trauma to the peripheral nerve at P10 [43]. In addition, the duration of vincristine treatment may be relevant to the clinical observation that ALL patients can receive vincristine at varying intervals over a period of several years [49]. Littermates injected with equivalent volumes of sterile saline served as a control group.

2.3 Behavioral testing

Response to mechanical stimuli—Mechanical reflex withdrawal thresholds were measured prior to each injection, and then at weekly intervals until P54, by an investigator blinded to treatment group. Briefly, rats (P11–P47) were lightly restrained on a flat surface and calibrated von Frey hair monofilaments were applied to the dorsal hindpaw [71], while at P54 rats were placed individually into clear acrylic containers separated by black opaque dividers and the von Frey hair filaments applied to the plantar surface of the hindpaw. Mechanical withdrawal thresholds were measured in all subjects according to the Simplified Up and Down (SUDO) method [8], with subsets of these rats undergoing additional behavioral tests of mechanical, heat and cold sensitivity. To assess dynamic mechanical allodynia, animals were first placed individually into clear acrylic containers separated by black opaque dividers and allowed to acclimate for at least 20 minutes. A paintbrush was used to stimulate the lateral plantar region of the hindpaw in the heel to toe direction. Responses were recorded on a scale of 0 – 3 and presented as an allodynia score, where 0 = no response or occasionally walking away; 1 = lifting the stimulated paw towards the body; 2 = strong lateral lifting of the paw above the level of the body; and 3 = shaking or licking the paw [17]. To obtain the average score for each animal, this test was repeated on each paw three times at 10 s intervals. For the pinprick test, a blunted needle was pressed against the plantar surface of the hindpaw without penetration, and repeated on each paw 3 times at one minute intervals [17]. The total number of withdrawals was counted for each rat and expressed as a percent response (based on a maximum of 6).

Response to heat stimuli—Sensitivity to noxious heat was assessed using the Hargreaves test [25]. Briefly, animals were first placed individually into clear acrylic

containers separated by black opaque dividers on the glass surface of the thermal testing apparatus (Model 336, IITC/Life Science Instruments, Woodland Hills, CA) and allowed to acclimate for at least 20 minutes. A mobile radiant heat source was directed to the mid-plantar surface of the hindpaw at 5 minute intervals per rat, alternating paws, for a total of 6 trials. The radiant heat beam was turned off automatically by a photocell feedback sensor upon paw withdrawal and the time was recorded. To avoid potential tissue damage, the cut-off time was set to 20 seconds. The mean withdrawal latency was calculated for each rat from the 6 trials. Withdrawal latencies were evaluated weekly starting at P26 until P54.

Response to cold stimuli—The cold plantar assay was used to evaluate noxious cold sensitivity [11] in a separate cohort of rats as was used in the tests for noxious heat sensitivity. Briefly, animals were first placed individually into clear acrylic containers separated by black opaque dividers that were set on top of 3/16" borosilicate glass (Stemmerich Inc., St. Louis, MO) and allowed to acclimate for 20 mins prior to testing. A dry ice pellet was applied to the hindpaw through the glass and the time until hindpaw withdrawal was recorded at 5 minute intervals per rat, alternating paws, for a total of 6 trials and the mean withdrawal latency was calculated. Withdrawal latencies were evaluated weekly starting at P20 until approximately 7 weeks of age.

Evaluation of gross motor function—Animals were placed on an accelerating rotarod treadmill set at a constant speed (4 r.p.m.). The rod was then accelerated from a rate of 4 to 40 r.p.m. over a 10 minute period. The animal's performance was recorded as the latency to fall (in seconds) and evaluated weekly starting after the last injection of VNC.

Evaluation of fine motor function—The foot fault test was used for skilled locomotor assessment [44]. On P20, animals were trained to cross a horizontal ladder with irregular rung spacing (ranging from 1 – 2 cm) from a neutral starting cage to reach their home cage. Testing consisted of two trials per animal and was performed weekly starting on P21 until P37. Two independent experimenters blinded to the treatment group rated each step on a 7-category scale based on the position and accuracy, as well as errors that occurred in paw placement, according to the following scoring criteria: 0 = total miss; 1 = deep slip; 2 = slight slip; 3 = replacement; 4 = correction; 5 = partial placement; 6 = correct placement. The scores from the two experimenters were then averaged and presented as percent accuracy.

2.4 Immunohistochemistry

At P25 and P33, animals were deeply anesthetized with sodium pentobarbital and transcardially perfused with 0.1M phosphate-buffered saline. The skin from the plantar surface of the hindpaw was removed and placed in 4% PFA for 4 hours, then transferred to 20% sucrose overnight at 4°C. Tissue was embedded in Tissue Freezing Medium (TFM, General Data Healthcare, Cincinnati, OH), sectioned at 40 µm using a cryostat (Leica), and processed as free-floating sections. Following a 1 hour incubation in blocking solution (0.1M phosphate-buffered saline containing 5% normal donkey serum/0.3% Triton X-100) at room temperature (RT), liquid was decanted and sections were incubated overnight at RT with the primary antibodies: PGP9.5 (Dako; rabbit; 1:500), and collagen IV (Southern

Biotech; goat; 1:500). The primary antibody was then decanted and sections were washed three times for 30 minutes each in wash buffer consisting of PBS + 0.3% Triton X-100. After the wash buffer was removed, the secondary antibodies donkey anti-rabbit Alexa 488 (1:500) and donkey anti-goat Alexa 594 (1:500; Jackson ImmunoResearch) were added for 30 minutes at RT. Following incubation with the secondary antibodies, the tissue was placed in wash buffer three times for 30 minutes each then mounted onto slides, cover-slipped, and stored at 4°C until imaging.

2.5 Quantification of intraepidermal nerve fibers (IENF)

For IENF quantification, three plantar skin sections were selected per animal and four fields of view per section were chosen at random by an experimenter blinded to the treatment group. Images were obtained using a Zeiss LSM 710 confocal microscope equipped with argon and helium neon lasers applying a 20× objective and captured as z-stacks of confocal optical sections at a thickness of 1 µm. Intraepidermal nerve fibers that crossed the collagen IV-stained dermal/epidermal junction into the epidermis were counted (as the number of fibers per square mm of epidermis; IENF/mm²), and fibers that branched after crossing the basement membrane were recorded as a single fiber. Langerhans cells (LC) that showed PGP9.5 immunoreactivity were counted if the cell body was visible. For each section, a mean IENF density and LC count was calculated from the four fields of view by two independent experimenters blinded to treatment group, and the results across experimenters were then averaged. For each experimental group, 4 – 6 rats were analyzed in this manner.

2.6 Statistical analysis

Behavioral data and IENF counts were analyzed using two-way analysis of variance (ANOVA) with Holm-Sidak *post hoc* tests (Prism; GraphPad Software; La Jolla, CA). Langerhans cell counts were compared between groups using the unpaired student's t-test. For all tests, the significance level was set at $p < 0.05$. *n* refers to the number of rats in a given group unless otherwise stated. All data are reported as mean ± SEM.

3. Results

3.1 Toxicity of vincristine administration in the developing rat

Beginning at postnatal day (P) 11, rats were injected i.p. with vincristine (15, 30, 45 or 60 µg/kg) or vehicle with a four day-on, two day-off, five day-on schedule (see Methods). The daily administration of vincristine (VNC) at 45 or 60 µg/kg routinely led to death of the animal following the third injection (*data not shown*). Therefore, to explore the effects of high VNC doses on sensorimotor function in the developing rat, we modified the dosing protocol such that VNC was administered at 60 µg/kg five times between P11 and P21 (see Methods). At this dose, VNC caused a slight, but significant, decrease in the rate of weight gain in both males ($F_{(8,433)} = 11.03$, $p < 0.0001$, $n = 18 - 27$; Fig. 1A) and females ($F_{(8,451)} = 7.40$, $p < 0.0001$, $n = 14 - 27$; Fig. 1B) beginning at 3 weeks of age. Nonetheless, weight loss was never observed and the animals were judged to be in good overall health. In support of this notion, VNC administration (60 µg/kg) had no effect on gross motor function at any time point examined, as there were no differences in latency to fall on the rotarod test between VNC-treated rats and vehicle-treated littermates ($p = 0.82$; Fig. 2A). Assessment of

fine motor skills was also carried out weekly using the foot fault test (see Methods). The high dose of VNC had no impact on foot fault accuracy scores compared to vehicle-treated controls ($p = 0.78$; Fig. 2B).

3.2 Vincristine administration during early life evokes delayed mechanical pain hypersensitivity

Mechanical withdrawal thresholds were measured at various postnatal ages using von Frey hair filaments following the administration of VNC or vehicle solution between P11 and P21 (see above). As described previously [29,66], mechanical thresholds steadily increased with age in males ($F_{(10,493)} = 147.56$, $p < 0.0001$, $n = 18 - 27$; Fig. 3A) and females ($F_{(10,457)} = 113.70$, $p < 0.0001$, $n = 14 - 27$; Fig. 3B) within both experimental groups. Low dose administration of VNC (15 or 30 $\mu\text{g/kg}$) failed to produce behavioral signs of neuropathic pain at any stage of postnatal development examined, as mechanical withdrawal thresholds were similar between VNC-treated and littermate controls injected with vehicle ($F_{(20,232)} = 1.15$, $p = 0.2976$, $n = 9$; Supp. Fig. 1). Withdrawal latencies to noxious heat were also unaltered by the administration of VNC at these doses ($F_{(8,70)} = 0.24$, $p = 0.9819$, $n = 7$; Supp. Fig. 2).

Meanwhile, early life exposure to VNC at 60 $\mu\text{g/kg}$ resulted in hypersensitivity to mechanical stimuli that emerged following the completion of the VNC regimen in both males ($F_{(10,493)} = 3.42$, $p = 0.0002$, $n = 18 - 27$ in each group; Fig. 3A) and females ($F_{(10,457)} = 6.35$, $p < 0.0001$, $n = 14 - 27$; Fig. 3B), as VNC-treated animals exhibited a significant decrease in mechanical withdrawal thresholds (~35%) beginning on P26 (i.e. 5 days after the final VNC injection) compared to vehicle-treated littermates (Fig. 3A, B). This persisted throughout the testing period, with mechanical withdrawal thresholds in the VNC groups remaining significantly lower than the vehicle groups at P54. Given that VNC evoked mechanical hypersensitivity with a similar onset and duration in males and females, data from both sexes were pooled for subsequent behavioral analyses.

To investigate the degree to which VNC during the early postnatal period induces dynamic mechanical allodynia in the developing rat, the prevalence of hindpaw withdrawals in response to innocuous brush was examined at multiple ages between P20 and P33. These time points were chosen to span the age at which static mechanical allodynia emerged (as measured by the administration of von Frey hairs). Importantly, VNC treatment significantly increased allodynia scores in response to a paintbrush stimulus ($F_{(1,134)} = 8.32$, $p = 0.0046$, $n = 19 - 26$ in each group), which was most evident at P26 ($p = 0.0038$; Holm-Sidak post-test for multiple comparisons; Fig. 4A). In addition, the frequency of hindpaw withdrawal to pinprick stimuli was significantly elevated by VNC exposure ($F_{(1,97)} = 12.00$, $p = 0.0008$, $n = 13 - 23$; Fig. 4B), suggesting that VNC treatment during early life also significantly increases the sensitivity to noxious mechanical stimuli.

3.3 Early vincristine treatment does not alter sensitivity to noxious thermal stimuli

Sensitivity to noxious radiant heat was examined weekly between P26 and P54 in rats treated with 60 $\mu\text{g/kg}$ VNC (or vehicle), corresponding to a period during which a clear hypersensitivity to mechanical stimulation was observed (Figs. 3, 4). There was no

significant effect of neonatal VNC administration on mean withdrawal latencies to noxious heat at any age examined ($F_{(1,145)} = 0.06$, $p = 0.81$, $n = 16$ in each group; Fig. 5A). Similarly, VNC treatment had no effect on the withdrawal latencies in response to noxious cold stimuli at any time point ($F_{(1,238)} = 0.01$, $p = 0.92$, $n = 25$; Fig. 5B), suggesting that early life VNC exposure selectively increases mechanical pain sensitivity in the developing rat.

3.4 Loss of intraepidermal nerve fibers following vincristine treatment during early life

Previous studies in both humans and rats have demonstrated vincristine- and paclitaxel-induced painful peripheral neuropathies are accompanied by a reduction of intraepidermal nerve fiber (IENF) density corresponding to the onset of mechanical hypersensitivity [5,9,10,47,57]. Based on these reports, we next sought to determine if the onset of mechanical allodynia was associated with changes in IENF innervation in the hindpaw skin. Vincristine treatment caused a statistically significant reduction of IENF density ($F_{(1,56)} = 5.616$, $p = 0.021$, $n = 12 - 18$ sections from 4 – 6 rats in each group; Fig. 6A–C). While there was no difference in the number of IENFs per mm^2 of the epidermal border between treatment groups at P25 ($p = 0.607$, Holm-Sidak post-test), VNC-treated rats exhibited significantly lower IENF counts relative to controls at P33 ($p = 0.047$, Holm-Sidak post-test; Fig. 6C, right). At antibody dilutions optimized for visualizing IENFs, PGP9.5 immunoreactivity was also observed in Langerhans cells (Fig. 6B), which are the resident immune cells of the skin [2,30,64]. As previously reported in the adult rat [57], the reduction in IENF density after VNC treatment was accompanied by an increase in the number of PGP9.5-positive Langerhans cells ($p = 0.017$, unpaired t-test, $n = 12$ sections from 4 rats in each group; Fig 6D).

4. Discussion

The major goal of this study was to establish the first preclinical model of pediatric chemotherapy-induced neuropathic pain resulting from the administration of vinca alkaloid derivatives during early life. The results clearly demonstrate that neonatal vincristine (VNC) exposure evokes mechanical pain hypersensitivity that emerges following the conclusion of treatment, persists into adulthood, and includes enhanced responses to both static and dynamic mechanical stimuli without accompanying changes in the sensitivity to noxious thermal stimulation or motor function.

In humans, symptoms of vincristine-induced peripheral neuropathy are often persistent and can sometimes worsen after the termination of chemotherapy or even emerge after drug withdrawal [48,50]. Interestingly, the mechanical hypersensitivity observed after VNC administration in the neonatal rat (as measured using von Frey hairs) failed to completely resolve during the time period examined and emerged with a delay (~5 days) relative to the cessation of the VNC regimen (Fig. 3). The increased responsiveness to a dynamic, innocuous mechanical stimulus (i.e. paintbrush; Fig. 4A) and a punctate, noxious mechanical stimulus (i.e. pinprick; Fig. 4B) also appeared following the termination of the VNC treatment, with a slightly longer delay in onset observed for the hypersensitivity to pinprick. Meanwhile, previous studies of VNC-induced CIPN in adult rats have reported an

earlier onset of mechanical allodynia (i.e. during the second week of treatment) even at lower doses (50 µg/kg; [72]) than used here, although the magnitude of the mechanical hypersensitivity was similar [1,3,46,62,63]. Importantly, recent preclinical work has clearly shown that peripheral nerve damage occurring before 3 weeks of age evokes neuropathic pain with a significant delay in onset relative to adult nerve injury [14,29,66], which has been ascribed to a shift from an anti-inflammatory to pro-inflammatory cytokine profile within the CNS which occurs during adolescence [43]. Interestingly, this delay appears to be unique to neuropathic pain, as neonatal rats exhibit robust inflammatory pain from the first days of life following the intraplantar injection of carrageenan or CFA [42,70].

Nonetheless, it should be noted that unlike models involving a discrete physical injury to the peripheral nerve, the precise time point at which the neonatal VNC administration causes axonal trauma is difficult to ascertain. While we first observed a reduction in IENF density at P33 (Fig. 6), it is possible that the fibers entered an early phase of degeneration well before this age. Future immunohistochemical or molecular analyses of neuronal stress markers in the DRG [63], and/or electrophysiological measurements of axonal conduction along primary afferent fibers [62,75], are needed to better pinpoint the onset of axonal dysfunction after early life VNC exposure. In addition, given that symptoms of vincristine-induced peripheral neuropathy can depend on cumulative dose [16,23], we cannot exclude the possibility that VNC doses greater than 60 µg/kg could result in an earlier emergence of mechanical hypersensitivity in the developing rat. While our dosage is lower than those employed in clinical practice to achieve antineoplastic activity [53], toxicity issues prevented the examination of higher VNC doses in our rodent model during this particular stage of development.

As is the case in adults, mounting evidence points to important sex differences in the prevalence and magnitude of pediatric pain, with females often exhibiting greater pain sensitivity in both the clinical and experimental settings [7,33,78]. However, we observed no striking differences in the time course or magnitude of mechanical hypersensitivity between male and female littermates after early VNC exposure as measured by von Frey hair testing. Distinct mechanisms could nonetheless underlie CIPN-related pain after neonatal VNC administration in males versus females. Indeed, recent work demonstrates that the key neuroimmune interactions driving neuropathic pain are sexually dimorphic with microglia reportedly playing a prominent role in males but not females [58,59], although it should be noted that the importance of microglial activation for the induction of CIPN-induced neuropathic pain has been questioned [77]. Interestingly, the mechanisms contributing to neuropathic pain may partly depend on the extent of peripheral nerve damage, as knockout of toll-like receptor 4 (TLR4) signaling abolishes tactile allodynia selectively in males after spinal nerve ligation [61] but prevents hypersensitivity in both sexes following cisplatin-induced polyneuropathy [74].

In contrast to the marked mechanical hypersensitivity in the aftermath of neonatal VNC administration, withdrawal latencies to noxious heat or cold stimuli were not influenced by VNC treatment during early life. Prior work in adult rats has produced mixed findings with regards to the degree of thermal hyperalgesia and cold allodynia produced by VNC, with heat sensitivity reportedly increased [1], decreased [3,63], or unchanged [46,72]. Similarly,

cold allodynia has been observed in response to VNC-induced neuropathy in some studies [3] but not others [46,72]. In humans, sensory testing has revealed VNC-induced impairments in temperature sensation. Adults with VNC-induced neuropathy are more sensitive to noxious cold stimuli and have elevated warm detection thresholds [16], while children are less likely to discern differences in temperature throughout the first year of VNC treatment when innocuous cold objects are applied to the skin [36,37]. Unfortunately, to our knowledge, sensitivities to noxious cold or heat stimuli have not been evaluated in the pediatric population following VNC treatment. Therefore, the degree to which the neonatal rat model fully recapitulates the clinical phenotype seen after pediatric VNC exposure remains unclear. For example, although motor dysfunction and loss of deep tendon reflexes are observed clinically in pediatric VNC-induced neuropathy [31,36,51,52,65], we did not observe alterations in either gross or fine motor skills in our experiments (Fig. 2).

Damage to peripheral nerve endings and Langerhans cell (LC) activation are implicated in chemoneuropathy as well as other neuropathic pain conditions such as post-herpetic neuralgia, diabetic neuropathy, and type-I complex regional pain syndrome [2,12,57]. VNC administration during early life significantly decreased epidermal innervation and led to the appearance of PGP9.5-positive Langerhans cells (Fig. 6), which have also been noted in adult rodent models of CIPN at the onset of hypersensitivity to mechanical stimuli [9,57]. LCs represent the population of epidermal dendritic cells closely associated with IENF terminals [22,30,55]. As the resident immune cells of the skin, LCs are responsible for antigen presentation, cytokine production, and pro-inflammatory receptor expression [2,30,64]. IENFs have been shown to regulate LC dynamics [30] and, in turn, LCs can regulate cutaneous innervation [15]. Although the functional significance of the potential interactions between IENFs and LCs in the context of CIPN remains unclear, it is possible that IENF loss may signal LC activation and subsequent cytokine release, resulting in the sensitization of the remaining nociceptive fibers or those in the process of degenerating. Indeed, A δ and C fibers from adult rats with CIPN exhibit increased spontaneous activity [3,62,75]. However, recent work demonstrates that the subtypes of primary afferent neurons whose excitability is altered by peripheral inflammation are dependent upon the age at which the insult occurs [32]. In this regard, it will ultimately be important to characterize the excitability of developing sensory neurons at various time points after neonatal VNC treatment.

It should also be noted that aberrant sensory input resulting from injury during the early postnatal period can have prolonged consequences for central nociceptive processing, including (but not limited to) a disruption in the balance between excitatory and inhibitory synaptic transmission and a facilitation of synaptic long-term potentiation (LTP) in the spinal dorsal horn [40,41], elevated spinal neuroimmune responses [4,67], heightened opioidergic tone in the brain [34,35] and changes in descending inhibitory pathways [68,76]. This highlights the need for future studies examining the effects of early life VNC exposure on the functional organization of developing pain networks within the CNS. Similarly, while mounting evidence from both rodents [20,39–41,54,66,68,71] and humans [26,27,69] suggests that neonatal injuries can ‘prime’ developing nociceptive pathways such that a subsequent injury evokes an exacerbated level of pain hypersensitivity, it remains to be determined whether neonatal VNC treatment modulates the behavioral response to an

unrelated injury later in life. Finally, while the present study investigated the effects of VNC administration to naïve rats as a means to isolate the effects of the drug itself, it will ultimately be critical to elucidate how neonatal VNC alters pain sensitivity and nociceptive circuitry when administered in the presence of ALL or other pediatric cancers, in order to better mimic the clinical scenario.

In conclusion, this work lays an essential foundation for future investigations into the neurobiological underpinnings of chemotherapy-induced neuropathic pain during early life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the U.S. National Institutes of Health grant NS080889 (MLB). The authors would like to thank Mrs. Elizabeth Serafin for her technical assistance and Drs. Judith Strong and Temugin Berta for their constructive feedback regarding this manuscript.

References

1. Aley KO, Reichling DB, Levine JD. Vincristine hyperalgesia in the rat: a model of painful vincristine neuropathy in humans. *Neuroscience*. 1996; 73(1):259–265. [PubMed: 8783247]
2. Asahina A, Hosoi J, Grabbe S, Granstein RD. Modulation of Langerhans cell function by epidermal nerves. *J Allergy Clin Immunol*. 1995; 96(6, Supplement):1178–1182. [PubMed: 8543775]
3. Authier N, Gillet J-P, Fialip J, Eschalier A, Coudore F. A New Animal Model of Vincristine-Induced Nociceptive Peripheral Neuropathy. *NeuroToxicology*. 2003; 24(6):797–805. [PubMed: 14637374]
4. Beggs S, Currie G, Salter MW, Fitzgerald M, Walker SM. Priming of adult pain responses by neonatal pain experience: maintenance by central neuroimmune activity. *Brain*. 2012; 135(2):404–417. [PubMed: 22102650]
5. Bennett GJ, Liu GK, Xiao WH, Jin HW, Siau C. Terminal arbor degeneration—a novel lesion produced by the antineoplastic agent paclitaxel. *Eur J Neurosci*. 2011; 33(9):1667–1676. [PubMed: 21395870]
6. Boada MD, Gutierrez S, Giffear K, Eisenach JC, Ririe DG. Skin incision-induced receptive field responses of mechanosensitive peripheral neurons are developmentally regulated in the rat. *J Neurophysiol*. 2012; 108(4):1122–1129. [PubMed: 22673323]
7. Boerner KE, Birnie KA, Caes L, Schinkel M, Chambers CT. Sex differences in experimental pain among healthy children: a systematic review and meta-analysis. *Pain*. 2014; 155(5):983–993. [PubMed: 24508752]
8. Bonin RP, Bories C, De Koninck Y. A simplified up-down method (SUDO) for measuring mechanical nociception in rodents using von Frey filaments. *Mol Pain*. 2014; 10:26. [PubMed: 24739328]
9. Boyette-Davis J, Xin W, Zhang H, Dougherty PM. Intraepidermal nerve fiber loss corresponds to the development of Taxol-induced hyperalgesia and can be prevented by treatment with minocycline. *Pain*. 2011; 152(2):308–313. [PubMed: 21145656]
10. Boyette-Davis JA, Cata JP, Driver LC, Novy DM, Bruel BM, Mooring DL, Wendelschafer-Crabb G, Kennedy WR, Dougherty PM. Persistent chemoneuropathy in patients receiving the plant alkaloids paclitaxel and vincristine. *Cancer Chemother Pharmacol*. 2013; 71(3):619–626. [PubMed: 23228992]
11. Brenner DS, Golden JP, Gereau RW. A Novel Behavioral Assay for Measuring Cold Sensation in Mice. *PLoS ONE*. 2012; 7(6):e39765. [PubMed: 22745825]

12. Casanova-Molla J, Morales M, Planas-Rigol E, Bosch A, Calvo M, Grau-Junyent JM, Valls-Sole J. Epidermal Langerhans cells in small fiber neuropathies. *Pain*. 2012; 153(5):982–989. [PubMed: 22361736]
13. Clancy B, Finlay BL, Darlington RB, Anand KJS. Extrapolating brain development from experimental species to humans. *Neurotoxicology*. 2007; 28(5)doi: 10.1016/j.neuro.2007.1001.1014
14. Costigan M, Scholz J, Woolf CJ. Neuropathic pain: a maladaptive response of the nervous system to damage. *Annu Rev Neurosci*. 2009; 32:1–32. [PubMed: 19400724]
15. Doss AL, Smith PG. Langerhans cells regulate cutaneous innervation density and mechanical sensitivity in mouse footpad. *Neurosci Lett*. 2014; 578:55–60. [PubMed: 24970748]
16. Dougherty PM, Cata JP, Burton AW, Vu K, Weng H-RR. Dysfunction in multiple primary afferent fiber subtypes revealed by quantitative sensory testing in patients with chronic vincristine-induced pain. *J Pain Symptom Manage*. 2007; 33(2):166–179. [PubMed: 17280922]
17. Duan B, Cheng L, Bourane S, Britz O, Padilla C, Garcia-Campmany L, Krashes M, Knowlton W, Velasquez T, Ren X, Ross SE, Lowell BB, Wang Y, Goulding M, Ma Q. Identification of Spinal Circuits Transmitting and Gating Mechanical Pain. *Cell*. 2014; 159(6):1417–1432. [PubMed: 25467445]
18. Fitzgerald M. Cutaneous primary afferent properties in the hind limb of the neonatal rat. *J Physiol*. 1987; 383:79–92. [PubMed: 3656143]
19. Fitzgerald M. The development of nociceptive circuits. *Nat Rev Neurosci*. 2005; 6(7):507–520. [PubMed: 15995722]
20. Fitzgerald M, Jennings E. The postnatal development of spinal sensory processing. *Proc Natl Acad Sci U S A*. 1999; 96(14):7719–7722. [PubMed: 10393887]
21. Fitzgerald M, McKelvey R. Nerve injury and neuropathic pain—A question of age. *Exp Neurol*. 2016; 275(Pt 2):296–302. [PubMed: 26220898]
22. Gaudillere A, Misery L, Souchier C, Claudy A, Schmitt D. Intimate associations between PGP9-5-positive nerve fibres and Langerhans cells. *Br J Dermatol*. 1996; 135(2):343–344. [PubMed: 8881702]
23. Grisold W, Cavaletti G, Windebank AJ. Peripheral neuropathies from chemotherapeutics and targeted agents: diagnosis, treatment, and prevention. *Neuro-oncology*. 2012; 14(Suppl 4):iv45–54. [PubMed: 23095830]
24. Gurney JG, Severson RK, Davis S, Robison LL. Incidence of cancer in children in the United States. Sex-, race-, and 1-year age-specific rates by histologic type. *Cancer*. 1995; 75(8):2186–2195. [PubMed: 7697611]
25. Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain*. 1988; 32(1):77–88. [PubMed: 3340425]
26. Hermann C, Hohmeister J, Demirakca S, Zohsel K, Flor H. Long-term alteration of pain sensitivity in school-aged children with early pain experiences. *Pain*. 2006; 125(3):278–285. [PubMed: 17011707]
27. Hohmeister J, Kroll A, Wollgarten-Hadamek I, Zohsel K, Demirakça S, Flor H, Hermann C. Cerebral processing of pain in school-aged children with neonatal nociceptive input: an exploratory fMRI study. *Pain*. 2010; 150(2):257–267. [PubMed: 20471751]
28. Holland JF, Scharlau C, Gailani S, Krant MJ, Olson KB, Horton J, Shnider BI, Lynch JJ, Owens A, Carbone PP, Colsky J, Grob D, Miller SP, Hall TC. Vincristine treatment of advanced cancer: a cooperative study of 392 cases. *Cancer Res*. 1973; 33(6):1258–1264. [PubMed: 4352365]
29. Howard RF, Walker SM, Mota MP, Fitzgerald M. The ontogeny of neuropathic pain: Postnatal onset of mechanical allodynia in rat spared nerve injury (SNI) and chronic constriction injury (CCI) models. *Pain*. 2005; 115(3):382–389. [PubMed: 15911165]
30. Hsieh ST, Choi S, Lin WM, Chang YC, McArthur JC, Griffin JW. Epidermal denervation and its effects on keratinocytes and Langerhans cells. *J Neurocytol*. 1996; 25(9):513–524. [PubMed: 8910797]
31. Jain P, Gulati S, Seth R, Bakhshi S, Toteja GS, Pandey RM. Vincristine-induced neuropathy in childhood ALL (acute lymphoblastic leukemia) survivors: prevalence and electrophysiological characteristics. *J Child Neurol*. 2014; 29(7):932–937. [PubMed: 23781018]

32. Jankowski MP, Ross JL, Weber JD, Lee FB, Shank AT, Hudgins RC. Age-dependent sensitization of cutaneous nociceptors during developmental inflammation. *Mol Pain*. 2014; 10:34. [PubMed: 24906209]
33. King S, Chambers CT, Huguet A, MacNevin RC, McGrath PJ, Parker L, MacDonald AJ. The epidemiology of chronic pain in children and adolescents revisited: a systematic review. *Pain*. 2011; 152(12):2729–2738. [PubMed: 22078064]
34. LaPrairie J, Murphy AZ. Neonatal injury alters adult pain sensitivity by increasing opioid tone in the periaqueductal gray. *Front Behav Neurosci*. 2009; 3(31)
35. LaPrairie JL, Murphy AZ. Long-term impact of neonatal injury in male and female rats: Sex differences, mechanisms and clinical implications. *Front Neuroendocrinol*. 2010; 31(2):193–202. [PubMed: 20144647]
36. Lavoie Smith EM, Li L, Chiang C, Thomas K, Hutchinson RJ, Wells EM, Ho RH, Skiles J, Chakraborty A, Bridges CM, Renbarger J. Patterns and severity of vincristine induced peripheral neuropathy in children with acute lymphoblastic leukemia. *J Peripher Nerv Syst*. 2015; 20(1):37–46. [PubMed: 25977177]
37. Lavoie Smith EM, Li L, Hutchinson RJ, Ho R, Burnette WB, Wells E, Bridges C, Renbarger J. Measuring vincristine-induced peripheral neuropathy in children with acute lymphoblastic leukemia. *Cancer Nurs*. 2013; 36(5):E49–60. [PubMed: 23842524]
38. LeClerc JM, Billett AL, Gelber RD, Dalton V, Tarbell N, Lipton JM, Barr R, Clavell LA, Asselin B, Hurwitz C, Schorin M, Lipshultz SE, Declerck L, Silverman LB, Cohen HJ, Sallan SE. Treatment of childhood acute lymphoblastic leukemia: results of Dana-Farber ALL Consortium Protocol 87-01. *J Clin Oncol*. 2002; 20(1):237–246. [PubMed: 11773175]
39. Li J, Baccei ML. Neonatal tissue injury reduces the intrinsic excitability of adult mouse superficial dorsal horn neurons. *Neuroscience*. 2014; 256:392–402. [PubMed: 24184978]
40. Li J, Baccei ML. Neonatal Tissue Damage Promotes Spike Timing-Dependent Synaptic Long-Term Potentiation in Adult Spinal Projection Neurons. *J Neurosci*. 2016; 36(19):5405–5416. [PubMed: 27170136]
41. Li J, Blankenship ML, Baccei ML. Deficits in glycinergic inhibition within adult spinal nociceptive circuits after neonatal tissue damage. *Pain*. 2013; 154(7):1129–1139. [PubMed: 23639821]
42. Marsh D, Dickenson A, Hatch D, Fitzgerald M. Epidural opioid analgesia in infant rats II: responses to carrageenan and capsaicin. *Pain*. 1999; 82(1):33–38. [PubMed: 10422657]
43. McKelvey R, Berta T, Old E, Ji R-R, Fitzgerald M. Neuropathic Pain Is Constitutively Suppressed in Early Life by Anti-Inflammatory Neuroimmune Regulation. *J Neurosci*. 2015; 35(2):457–466. [PubMed: 25589741]
44. Metz GA, Whishaw IQ. Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: a new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. *J Neurosci Methods*. 2002; 115(2):169–179. [PubMed: 11992668]
45. Moss A, Beggs S, Vega-Avelaira D, Costigan M, Hathway GJ, Salter MW, Fitzgerald M. Spinal microglia and neuropathic pain in young rats. *Pain*. 2007; 128(3):215–224. [PubMed: 17110040]
46. Nozaki-Taguchi N, Chaplan SR, Higuera ES, Ajakwe RC, Yaksh TL. Vincristine-induced allodynia in the rat. *Pain*. 2001; 93(1):69–76. [PubMed: 11406340]
47. Peleshok JC, Ribeiro-da-Silva A. Delayed reinnervation by nonpeptidergic nociceptive afferents of the glabrous skin of the rat hindpaw in a neuropathic pain model. *J Comp Neurol*. 2011; 519(1):49–63. [PubMed: 21120927]
48. Postma TJ, Benard BA, Huijgens PC, Ossenkoppele GJ, Heimans JJ. Long-term effects of vincristine on the peripheral nervous system. *J Neurooncol*. 1993; 15(1):23–27. [PubMed: 8384253]
49. Pui C-H, Evans WE. Treatment of acute lymphoblastic leukemia. *N Engl J Med*. 2006; 354(2):166–178. [PubMed: 16407512]
50. Quasthoff S, Hartung HP. Chemotherapy-induced peripheral neuropathy. *J Neurol*. 2002; 249(1):9–17. [PubMed: 11954874]
51. Ramchandren S, Leonard M, Mody RJ, Donohue JE, Moyer J, Hutchinson R, Gurney JG. Peripheral neuropathy in survivors of childhood acute lymphoblastic leukemia. *J Peripher Nerv Syst*. 2009; 14(3):184–189. [PubMed: 19909482]

52. Reinders-Messelink HA, Schoemaker MM, Hofte M, Göeken LNH, Kingma A, van den Briel MM, Kamps WA. Fine motor and handwriting problems after treatment for childhood acute lymphoblastic leukemia. *Med Pediatr Oncol*. 1996; 27(6):551–555. [PubMed: 8888816]
53. Reiter A, Schrappe M, Ludwig WD, Hiddemann W, Sauter S, Henze G, Zimmermann M, Lampert F, Havers W, Niethammer D, Odenwald E, Ritter J, Mann G, Welte K, Gadner H, Riehm H. Chemotherapy in 998 unselected childhood acute lymphoblastic leukemia patients. Results and conclusions of the multicenter trial ALL-BFM 86. *Blood*. 1994; 84(9):3122–3133. [PubMed: 7949185]
54. Ren K, Anseloni V, Zou SPP, Wade EB, Novikova SI, Ennis M, Traub RJ, Gold MS, Dubner R, Lidow MS. Characterization of basal and re-inflammation-associated long-term alteration in pain responsivity following short-lasting neonatal local inflammatory insult. *Pain*. 2004; 110(3):588–596. [PubMed: 15288399]
55. Rice FL, Kinnman E, Aldskogius H, Johansson O, Arvidsson J. The innervation of the mystacial pad of the rat as revealed by PGP 9.5 immunofluorescence. *J Comp Neurol*. 1993; 337(3):366–385. [PubMed: 8282848]
56. Schorin MA, Blattner S, Gelber RD, Tarbell NJ, Donnelly M, Dalton V, Cohen HJ, Sallan SE. Treatment of childhood acute lymphoblastic leukemia: results of Dana-Farber Cancer Institute/Children's Hospital Acute Lymphoblastic Leukemia Consortium Protocol 85-01. *J Clin Oncol*. 1994; 12(4):740–747. [PubMed: 8151317]
57. Siau C, Xiao W, Bennett GJ. Paclitaxel-and vincristine-evoked painful peripheral neuropathies: loss of epidermal innervation and activation of Langerhans cells. *Exp Neurol*. 2006; 201(2):507–514. [PubMed: 16797537]
58. Sorge RE, LaCroix-Fralish ML, Tuttle AH, Sotocinal SG, Austin JS, Ritchie J, Chanda ML, Graham AC, Topham L, Beggs S, Salter MW, Mogil JS. Spinal cord Toll-like receptor 4 mediates inflammatory and neuropathic hypersensitivity in male but not female mice. *J Neurosci*. 2011; 31(43):15450–15454. [PubMed: 22031891]
59. Sorge RE, Mapplebeck JC, Rosen S, Beggs S, Taves S, Alexander JK, Martin LJ, Austin JS, Sotocinal SG, Chen D, Yang M, Shi XQ, Huang H, Pillon NJ, Bilan PJ, Tu Y, Klip A, Ji RR, Zhang J, Salter MW, Mogil JS. Different immune cells mediate mechanical pain hypersensitivity in male and female mice. *Nat Neurosci*. 2015; 18(8):1081–1083. [PubMed: 26120961]
60. Stanulla M, Schrappe M. Treatment of Childhood Acute Lymphoblastic Leukemia. *Semin Hematol*. 2009; 46(1):52–63. [PubMed: 19100368]
61. Stokes JA, Cheung J, Eddinger K, Corr M, Yaksh TL. Toll-like receptor signaling adapter proteins govern spread of neuropathic pain and recovery following nerve injury in male mice. *J Neuroinflammation*. 2013; 10:148. [PubMed: 24321498]
62. Tanner KD, Reichling DB, Levine JD. Nociceptor hyper-responsiveness during vincristine-induced painful peripheral neuropathy in the rat. *J Neurosci*. 1998; 18(16):6480–6491. [PubMed: 9698336]
63. Thibault K, Rivals I, M'Dahoma S, Dubacq S, Pezet S, Calvino B. Structural and molecular alterations of primary afferent fibres in the spinal dorsal horn in vincristine-induced neuropathy in rat. *J Mol Neurosci*. 2013; 51(3):880–892. [PubMed: 23975629]
64. Torii H, Yan Z, Hosoi J, Granstein RD. Expression of neurotrophic factors and neuropeptide receptors by Langerhans cells and the Langerhans cell-like cell line XS52: further support for a functional relationship between Langerhans cells and epidermal nerves. *J Invest Dermatol*. 1997; 109(4):586–591. [PubMed: 9326395]
65. Vainionpää L, Kovala T, Tolonen U, Lanning M. Vincristine therapy for children with acute lymphoblastic leukemia impairs conduction in the entire peripheral nerve. *Pediatr Neurol*. 1995; 13(4):314–318. [PubMed: 8771166]
66. Vega-Avelaira D, McKelvey R, Hathway G, Fitzgerald M. The emergence of adolescent onset pain hypersensitivity following neonatal nerve injury. *Mol Pain*. 2012; 8:30. [PubMed: 22531549]
67. Walker SM, Beggs S, Baccei ML. Persistent changes in peripheral and spinal nociceptive processing after early tissue injury. *Exp Neurol*. 2016; 275(Pt 2):253–260. [PubMed: 26103453]
68. Walker SM, Fitzgerald M. Surgical injury in the neonatal rat alters the adult pattern of descending modulation from the rostroventral medulla. *Anesthesiology*. 2015; 122(6):1391–1400. [PubMed: 25871742]

69. Walker SM, Franck LS, Fitzgerald M, Myles J, Stocks J, Marlow N. Long-term impact of neonatal intensive care and surgery on somatosensory perception in children born extremely preterm. *Pain*. 2009; 141(1–2):79–87. [PubMed: 19026489]
70. Walker SM, Meredith-Middleton J, Cooke-Yarborough C, Fitzgerald M. Neonatal inflammation and primary afferent terminal plasticity in the rat dorsal horn. *Pain*. 2003; 105(1–2):185–195. [PubMed: 14499435]
71. Walker SM, Tochiki KK, Fitzgerald M. Hindpaw incision in early life increases the hyperalgesic response to repeat surgical injury: critical period and dependence on initial afferent activity. *Pain*. 2009; 147(1–3):99–106. [PubMed: 19781855]
72. Weng HRR, Cordella JV, Dougherty PM. Changes in sensory processing in the spinal dorsal horn accompany vincristine-induced hyperalgesia and allodynia. *Pain*. 2003; 103(1–2):131–138. [PubMed: 12749967]
73. Windebank AJ, Grisold W. Chemotherapy-induced neuropathy. *J Peripher Nerv Syst*. 2008; 13(1): 27–46. [PubMed: 18346229]
74. Woller SA, Corr M, Yaksh TL. Differences in cisplatin-induced mechanical allodynia in male and female mice. *Eur J Pain*. 2015; 19(10):1476–1485. [PubMed: 25716290]
75. Xiao WH, Bennett GJ. Chemotherapy-evoked neuropathic pain: Abnormal spontaneous discharge in A-fiber and C-fiber primary afferent neurons and its suppression by acetyl-L- carnitine. *Pain*. 2008; 135(3):262–270. [PubMed: 17659836]
76. Zhang YH, Wang XM, Ennis M. Effects of neonatal inflammation on descending modulation from the rostroventromedial medulla. *Brain Res Bull*. 2010; 83(1–2):16–22. [PubMed: 20638459]
77. Zheng FY, Xiao WH, Bennett GJ. The response of spinal microglia to chemotherapy-evoked painful peripheral neuropathies is distinct from that evoked by traumatic nerve injuries. *Neuroscience*. 2011; 176:447–454. [PubMed: 21195745]
78. Zohsel K, Hohmeister J, Oelkers-Ax R, Flor H, Hermann C. Quantitative sensory testing in children with migraine: preliminary evidence for enhanced sensitivity to painful stimuli especially in girls. *Pain*. 2006; 123(1–2):10–18. [PubMed: 16495010]

Figure 1. Vincristine (VNC) treatment significantly reduces weight gain in both sexes. (A)

In male rats treated with VNC, there is a significant reduction in weight gain starting at P21 which persisted until the end of testing ($F_{(8,433)} = 11.03$, $p < 0.0001$, $n = 18 - 27$, 2-way ANOVA). **(B)** In females, VNC significantly attenuates weight gain starting at P19 ($F_{(8,451)} = 7.40$, $p < 0.0001$, $n = 14 - 27$, 2-way ANOVA; * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$; Holm-Sidak *post hoc* analysis). Each rat received one intraperitoneal injection (arrow) on P11, P13, P17, P19, and P21.

Figure 2. Neonatal vincristine administration does not impact gross or fine motor function in the developing rat. (A)

VNC treatment does not significantly affect gross motor function compared to vehicle-treated littermate controls on the rotarod test ($F_{(1,192)} = 0.18$, $p = 0.82$, $n = 17$, 2-way ANOVA). **(B)** Assessment of fine motor function demonstrated no difference in performance on the foot fault test between vehicle and VNC treated animals ($F_{(1,76)} = 0.08$, $p = 0.78$, $n = 10 - 16$ in each group, 2-way ANOVA).

Figure 3. Neonatal VNC exposure results in static mechanical allodynia by P26 in both sexes
 VNC treatment produces a delayed reduction in mechanical withdrawal thresholds in response to von Frey hair (VFH) application in both (A) male and (B) female rats compared to vehicle controls. Male VFH: ($F_{(10,493)} = 3.42$, $p = 0.0002$, $n = 18 - 27$ in each group); Female VFH: ($F_{(10,457)} = 6.35$, $p < 0.0001$, $n = 14 - 27$, 2-way ANOVA; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; Holm-Sidak *post hoc* analysis). Each rat received one intraperitoneal injection (arrow) on P11, 13, 17, 19, and 21.

Figure 4. Early life VNC produces dynamic mechanical allodynia and static mechanical hyperalgesia. (A)

Plot of allodynia score of hindpaw withdrawal in response to a paintbrush stimulus as a function of postnatal age following neonatal VNC treatment. A significant main effect of VNC treatment was observed when compared to vehicle-treated littermate controls ($F_{(1,134)} = 8.32$, $p = 0.0046$, $n = 19 - 26$ in each group, 2-way ANOVA; ** $p < 0.01$; Holm-Sidak *post hoc* analysis), suggesting the presence of dynamic mechanical allodynia after VNC. **(B)** Plot of frequency of hindpaw withdrawal in response to a pinprick stimulus at different ages after early VNC exposure. A significant main effect of VNC treatment was observed when compared to vehicle-treated littermate controls ($F_{(1,97)} = 12.00$, $p = 0.0008$, $n = 13 - 23$, 2-way ANOVA; *** $p < 0.001$; Holm-Sidak *post hoc* analysis).

Figure 5. VNC during the early postnatal period does not significantly affect withdrawal thresholds to noxious heat or cold. (A)

VNC did not significantly impact withdrawal latencies to noxious heat ($F_{(1,145)} = 0.06$, $p = 0.81$, $n = 16$, 2-way ANOVA). **(B)** VNC also failed to alter withdrawal latencies to noxious cold stimuli ($F_{(1,238)} = 0.01$, $p = 0.92$, $n = 25$, 2-way ANOVA).

Figure 6. Neonatal VNC treatment leads to decreased epidermal innervation and the presence of PGP9.5-positive Langerhans cells at P33

Confocal images of representative plantar skin sections immunostained for PGP9.5 (*green*) and collagen IV (*red*) from vehicle and VNC treated animals. On P33, VNC treated animals (**B**) had fewer PGP9.5-stained intraepidermal nerve fibers compared to those treated with saline (**A**). Arrows point to faintly stained Langerhans cells. Scale bar = 50 µm. (**C**) VNC leads to a reduction in PGP9.5-immunoreactive intraepidermal nerve fibers (IENF) two weeks after the end of treatment ($F_{(1,56)} = 5.616$, $p = 0.021$, 2-way ANOVA, $*p < 0.05$; Holm-Sidak *post hoc* analysis). (**D**) VNC leads to an increased presence of PGP9.5-positive Langerhans cells at P33 ($*p = 0.017$, unpaired t-test).